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(54) Title: BIOCOMPOSITE COMPRISING A MICROORGANISM AND AN ADDITIVE IN A FORMULATION MATRIX FOR BIOREMEDIATION AND POLLUTION CONTROL

(57) Abstract

The present invention relates to the development of technologies and strategies for using bacterial strains in bioremediation and pollution control. More particularly, the present invention relates to the establishment of several formulation technologies suitable for the production and storage of organic contaminant-degrading bacteria, and their use in biodegradation in the aqueous phase (surface water, groundwater), soil (slurry, composting, landfarming), and sediment (composting, in-situ treatment). The formulation technologies include encapsulation or immobilization of the microbes using a polyvinyl alcohol (PVA), vermiculite, or polyurethane matrix. Additives, including nutrients, densification agents, or adsorbents can also be co-encapsulated or co-immobilized therewith.

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DESCRIPTION

BIOCOMPOSITE COMPRISING A MICROORGANISM AND AN ADDITIVE IN A FORMULATION MATRIX FOR BIOREMEDIATION AND POLLUTION CONTROL

Cross-Reference to a Related Application

This application is a continuation-in-part of co-pending application Serial No. 08/037,215, filed March 26, 1993, which is a continuation-in-part of application Serial No. 07/913,274, filed July 14, 1992, now U.S. Patent No. 5,252,825.

Background of the Invention

Biodegradation technologies employing specially selected microorganisms capable of utilizing high molecular weight (HMW) polycyclic aromatic hydrocarbons (PAH) as a sole source of carbon and energy have proved their effectiveness toward bioremediation of soils contaminated with wood preservatives (creosote and pentachlorophenol) and related wastes (i.e., contaminated sludge). However, a major limitation associated with the transfer of these technologies from the laboratory to the field relates to the reliable introduction, or application, of viable, catabolically active microorganisms. Hence, there is a dire need for developing production, storage, and application strategies for these biocatalysts to facilitate site remediation. Although technologies such as microbial encapsulation and immobilization have previously been applied to water-soluble, readily biodegradable organics, these have been unsuccessfully applied to the biodegradation of high molecular weight polycyclic aromatic hydrocarbons (HMW PAHs). Alginate, a hydrogel matrix, has been unsuccessfully applied for use with PAH-degrading microorganisms. Bashan reported that when alginate was used to prepare microbial capsules, 8 log reduction in cell viability was observed during the encapsulation and drying (Bashan, Y. [1986] *Appl. Environ. Microbiol.* 51:1089-1098).

Encapsulation is defined as the inclusion of microbial cells in a matrix (i.e., capsule) that are released when needed. Release of the encapsulated microbial cells may be controlled by several mechanisms, including water dissolution of the matrix

material. Cell immobilization fixes microbial cells within a matrix or on a support surface (i.e., carrier) where biodegradation can occur.

Several encapsulation/immobilization techniques have been described using certain microorganisms for bioremediation of soils, sediments, and aqueous systems (Graham-Weiss, L., M. Bennett, A.S. Paau [1987] *Appl. Environ. Microbiol.* 53:2138-2140; O'Reilly, K.T., R.L. Crawford [1989] *Appl. Environ. Microbiol.* 9:2113-2118; Lin, J.E., H.Y. Wang, R.F. Hickey [1991] *Biotechnol. Bioengineer.* 38:273-179; Lin, J.E., H.Y. Wang [1991] *J. Ferm. Bioengineer.* 4:311-314; Baker, C.A., A.A. Brooks, R.Z. Greenley, J.M.S. Heins, EPA 0 320 483;). These techniques include polyvinyl alcohol (PVA) capsules for controlled release biocomposites, vermiculite formulation, and polyurethane (PU) pellets for polymer-immobilized biocomposites, for solid-particle-supported biocomposites. These methods are highly dependent on the following factors: (1) solubility of target compounds; (2) treatment purposes (remediation or prevention); and (3) properties of treated matrices and treatment processes. Viability and degrading activity of encapsulated/immobilized microorganisms are also two important parameters because, as an inoculant carrier for bioremediation, encapsulated/immobilized cells are required to possess an acceptable shelf life. The degrading activity per unit viable cells during field application determines the efficiency of the process.

The HMW PAH compounds have a very low water solubility. Hence, these compounds are usually absorbed onto soils and other solid matrices. Soil slurry reactors (Mueller, J.G., S.E. Lentz, B.O. Blattman, P.J. Chapman [1991] *Environ. Sci. Technol.* 25:1055-1061), landfarming, and composting represent common approaches to dealing with soil contaminated by these compounds. Bioreactors are, in general advantageous as compared to these bioremediation approaches (e.g., composting, land farming, and *in situ* treatment) because the physicochemical variables (e.g., pH, nutrient concentrations, biomass, oxygen-transfer rate, contaminant loading rate, etc.) of a bioreactor can be precisely controlled. Conditions in a bioreactor can be optimized for the desired microbial activities in order to maximize performance. However, previous attempts to apply bioreactor technologies to the treatment of soil and water contaminated wit the chemicals found in organic wood preservatives have often proven unsuccessful (Dooley-Dana, M., M. Findley [1989] *Abstracts*, American

Society for Microbiology, Annual Meeting, May 14-18, 1989, New Orleans, LA, p. 363; Mahaffey, W., R. Sanford, A Strehler, A. Bourquin, *Id.* at 338; Mueller, J.G., S.E. Lantz, B.O. Blattmann, P. Chapman [1991] *J. Environ. Sci. Technol.* 25:1055-1061; Mueller, J.G., D.P. Middaugh, S.E. Lantz. P.J. Chapman [1991] *J. Appl. Environ. Microbiol.* 57:1277-1285; van der Hoek, J.P., L.G. Urlings, C.M. Grobben [1989] *Environ. Technol. Lett.* 10:185-194; Webb, O.F., T.L. Phelps, P.R. Bienkowski, P. DiGrazia, G.D. Reed, B. Applegate, D.C. White, G.S. Sayler [1991] *J. Appl. Biochem. Biotechnol.* 28/29:5-19).

Brief Summary of the Invention

The subject invention pertains to a unique formulation technology utilizing a microorganism with a capacity for degradation of an organic contaminant, e.g., a HMW, PAH or an organic pesticide. Advantageously, the storage property and degrading activity of the microorganism is superior using the subject encapsulation and immobilization techniques.

The technology includes techniques for formulating a microorganism in an encapsulation or immobilization matrix. These include PVA encapsulation, vermiculite formulation, and PU immobilization can be used. Exemplified herein are biocomposites of PAH-degradative *Pseudomonas* strains and a pesticide-degrading *Alcaligenes* strain. It would be further understood by a person of ordinary skill in the art that other microbial strains, including *Mycobacterium* strains, can also be used.

It is a further object of the invention that additives, e.g., adsorbents, nutrients, or densification agents can be included with the encapsulated or immobilized contaminant-degrading microorganism. For example, polyurethane-immobilized cells and co-immobilized cells and additives (adsorbents, nutrients, and densification agents) can be used according to the subject invention to degrade contaminants in aqueous media without a significant effect on degrading activity. By co-immobilizing slow-release formulations of nutrients in a polymer matrix, a major part of the nutrients can be provided to only the target microorganism, thereby reducing negative effects on the environment.

The subject invention further concerns a method for bioremediation of contaminated soil, air or water comprising contacting a novel biocomposite as

described by the subject invention with the contaminated soil, air, or water. This can uniquely be applicable to the combination of a groundwater circulation well with an immobilized-cell bioreactor for *in situ* bioremediation of contaminated soil and groundwater. An immobilized-cell biocatalyst containing specially selected microorganisms, and additives, e.g., adsorbents, density agents, and nutrients, can be accommodated in the bioreactor and integrated into the groundwater circulation process.

Brief Description of the Drawings

Figure 1 shows phenanthrene mineralization by free and PVA-encapsulated strain CRE 7 in SIU soil slurry (sterile).

Figure 2 shows fluoranthene mineralization by free and PVA-encapsulated strain EPA 505 in SIU soil slurry (sterile).

Figure 3 shows phenanthrene mineralization by free and PVA-encapsulated strain CRE 7 in SIU soil slurry (sterile) under a solid-state condition.

Figure 4 shows fluoranthene mineralization by free and PVA-encapsulated strain EPA 505 in SIU soil slurry (sterile) under a solid-state condition.

Figure 5 shows phenanthrene mineralization by free and vermiculite-carried strain CRE 7 in SIU soil slurry (sterile).

Figure 6 shows fluoranthene mineralization by free and vermiculite-carried strain EPA 505 in SIU soil slurry (sterile).

Figure 7 shows phenanthrene mineralization by free and vermiculite-carried strain CRE 7 in SIU soil slurry (sterile) under a solid-state condition.

Figure 8 shows fluoranthene mineralization by free and vermiculite-carried strain EPA 505 in SIU soil slurry (sterile) under a solid-state condition.

Figure 9 shows mineralization profiles of fluoranthene by non-immobilized and polyurethane-immobilized cells of strain EPA 505 with different adsorbents.

Figure 10 shows mineralization profiles of fluoranthene by polyurethane-immobilized cells of strain EPA 505 with encapsulated or external nitrogen and phosphate sources.

Figure 11 shows the effect of densification agents on fluoranthene mineralization by polyurethane-immobilized strain EPA 505.

Figure 12 shows a groundwater circulation well with an integrated immobilized-cell bioreactor.

Detailed Disclosure of the Invention

The subject invention concerns the use of formulation techniques, e.g., encapsulation or immobilization techniques, including polyvinyl alcohol (PVA) encapsulation, vermiculite formulation, and polyurethane immobilization, for enhancing the biodegradation of organic environmental contaminants including high molecular weight polycyclic aromatic hydrocarbon (HMW PAH) compounds. A microorganism so encapsulated or immobilized is termed a biocomposite. Other constituents can also be included in these biocomposites. The biocomposites prepared with these techniques can be selectively used in treating soil (or other solids), water, and vapor phase. The subject invention concerns organic contaminant-degrading, e.g., PAH-degrading, microorganisms encapsulated or immobilized using the techniques described. The microorganisms include, but are not limited to: (1) *Pseudomonas* sp. strain CRE 7 (a phenanthrene degrader); and (2) *Pseudomonas paucimobilis* strain EPA 505 (a fluoranthene degrader). In addition, the pesticide-degrading microorganism *Alcaligenes eutrophus* (2,4-D degrader), exemplified herein, was used. It would be understood by persons of ordinary skill in the art that other microbial strains are also capable of being used according to the subject invention. With PVA encapsulation of degradative microorganisms according to the subject invention, viability remains high during the preparation process. PVA-encapsulated cells can be stored at 4RC for at least two months with less than 2 log reduction in their viability. Vermiculite-carried strain CRE 7 can result in less than 1 log reduction in viability when stored at room temperature for two months. Viability of strain EPA 505 reduced only by about 3 log under the same conditions. Polyurethane encapsulation can also be used without significant loss of viability. The PVA-encapsulated and vermiculite-carried PAH-degrading microorganisms are effective under both slurry-phase and solid-phase conditions. The PVA capsules and vermiculite carriers can advantageously be stored for an extended period of time and they are also, advantageously, easily distributed. These two preparations of inoculants can maintain high degrading activity,

and provide slow-release or continuous cells. Thus, these two techniques can be used to treat PAH-contaminated soils and solids.

The subject invention also pertains to the combination of a groundwater circulation well with an immobilized cell bioreactor used, e.g., for *in situ* bioremediation of contaminated soil and groundwater. See Figure 12. This combined system has not been previously described to be successful with HMW PAHs or similar compounds. The subject invention employs an immobilized-cell biocatalyst, specially-selected contaminant-degraders, adsorbents, density agents, or nutrients in the bioreactor which can be integrated into the groundwater circulation process. The groundwater circulation technology produces a groundwater convection cell in the aquifer around the remediation well. The circulating groundwater continuously transports contaminants to the well to be contacted by the biocomposite in the bioreactor. When flowing through the well, the contaminants are adsorbed onto the immobilized cells inside the bioreactor and simultaneously biodegraded.

The subject invention further pertains to the co-immobilization of powdered diatomaceous earth with the microorganisms in a polyurethane matrix. The advantages of using diatomaceous earth are at least three-fold. First, diatomaceous earth can be used as an adsorbent. Advantageously, the compound adsorbed on the diatomaceous earth powder can be more readily desorbed and made available to the organisms, due to the lower binding capacity of diatomaceous earth. Second, the use of diatomaceous earth can increase the mechanical strength of polyurethane pellets. Third, the use of diatomaceous earth can provide more surface to accommodate cells.

In one embodiment of the subject invention, co-encapsulation of unique, slow-release nitrogen and phosphorous sources are used with organisms in the polyurethane matrix. Specifically, these unique phosphorous and nitrogen sources can be soybean lecithin and phenylacetylurea, respectively. These two substances have low water solubilities, and thus advantageously meet the need for the slow release of the nutrients, thereby, supporting the bioremediation by the immobilized cells. Density agents can also be co-encapsulated with organisms in the polyurethane matrix. Furthermore, it was determined that silica was a suitable density agent for the purposes of bioremediation.

The cells co-immobilized in a polyurethane matrix with a density agent, e.g., silica, can also be used in an *in situ* treatment process. Polyurethane immobilization technique provides reusable inoculants (without wash-out of the cells) in a continuous operating system. This immobilization technology, in conjunction with bioreactors, can be used in treating aqueous systems and vapor phases. This approach can effect several clean-up mechanisms, including soil flushing, adsorption, and eventually biodegradation in both the bioreactor and the contaminated matrix. Treating soils and groundwater contaminated with high molecular weight polycyclic aromatic hydrocarbons can be achieved with these specially designed immobilized cells.

Cold-water soluble PVA can also be used to encapsulate contaminant-degrading cells, including PAH-degrading microorganisms. Various additives, including nutrients and electron acceptors, can also be co-encapsulated in the PVA. Examples of nutrients which can be co-encapsulated include inorganic and organic nutrients (as nitrogen and phosphorous sources), including inorganic nitrogen and phosphate, skim milk, fish protein, yeast extract, and Bio2, with the degradative organisms in the PVA capsules. Furthermore, skim milk and yeast extract were an unpredictably good nutrient source in terms of supporting the cell viability and the biodegradation. Dissolution of the PVA capsules, and thus release of the encapsulated materials, can be controlled by manipulating the amount of water used, mode of water addition, or by varying the materials blended with PVA or by using PVAs with different molecular weights. Controlled release of the microbe or the co-encapsulated material using PVA-encapsulated PAH-degrading cells can advantageously reduce the need for repeated inoculation which can be required in certain bioremediation projects due to the inhibitory effects of indigenous microbes or hazardous environments.

The PVA-encapsulated cells were successfully used in soil (sediment) slurry bioreactors and soil (sediment) composting and landfarming processes. In soil slurry bioreactors, the PVA capsules dissolve rapidly and release the inoculants for biodegradation. In soil composting or landfarming, the PVA-encapsulated cells can be slowly released by adding a given amount of water and by using suitable agitation modes. The timed-release, or controlled-release, of biodegradation components can thus be effective for managed bioremediation and pollution prevention.

The PVA and vermiculite technologies of the subject invention were used to produce, store, and distribute the PAH-degrader EPA505 to a treatment system or contaminated site. Vermiculite-carried organisms e.g., EPA505, were used in soil (sediment) slurry bioreactors and soil (sediment) composting and landfarming processes. The viability of vermiculite-carried microorganisms can advantageously be maintained at room temperature.

Materials and Methods

Microorganisms and culture procedures. *Pseudomonas* sp. strain CRE 7 (phenanthrene degrader, originally designated as strain FAE 7; Mueller, J.G., P.J. Chapman, P.H. Pritchard [1989] *Appl. Environ. Microbiol.* 55:3085-3090) and *Pseudomonas paucimobilis* strain EPA 505 (fluoranthene degrader described in U.S. Patent Nos. 5,132,224 and 5,252,825, which are hereby incorporated by reference) were used for encapsulation or immobilization. These two microorganisms were grown in LB broth under conditions well known in the art, concentrated by centrifugation, and resuspended in phosphate buffer (0.025 M). The resultant cell concentrate was used in these procedures.

Encapsulation using polyvinyl alcohol (PVA). Ten parts (wt.) PVA (cold-water soluble, MW 30,000-70,000; Sigma Chemical Co., St. Louis, MO), 2 parts Kaolin, and 1 part dextrose were mixed with 100 parts of distilled water. The mixture was stirred for about 40 minutes. The pH of the mixture was adjusted to approximately 7 with 0.1 N NaOH solution. The finished solution was autoclaved at 121RC for 20 minutes and then stored at room temperature. To prepare PVA capsules, hexane (100 ml in a 250 ml flask) was cooled at −70RC for at least 30 minutes. Two milliliters of the PVA solution and 0.5 ml of cell concentrate were mixed in a test tube. The mixture was transferred into a 3 ml syringe. Droplets of the mixture were dropped through a needle (20G11/2) into 100 ml of the cold hexane. After the hexane was discarded, the formed capsules were dried in a freeze dryer for about 15 hours. Co-encapsulating an additive within the PVA matrix was achieved by standard procedures. Koalin (aluminum silicate) was also incorporated into the PVA capsule.

Preparation of vermiculite carriers. Vermiculite (Grade 3; Aldrich Chemical Co., Milwaukee, WI) was ground in a Willey mill. Vermiculite particles (100 mesh;

2 g) and LB broth (3 ml) were added to a flask. The flask was autoclaved at 121RC for 30 minutes. The vermiculite with the growth medium was then inoculated with a desired amount of liquid inoculum (*ca.* 10^7 cells/ml). The inoculated vermiculite was then incubated and stored at room temperature until used.

Immobilization and co-immobilization using polyurethane (PU). Two and one-half grams of polyurethane prepolymer (HYPOL 2000; Hampshire Controls Corp., Dover, NH) was cooled on ice and mixed with 1.5 ml of phosphate buffer (0.025 M). The mixture was stirred well for about 1 minute. Another 1.5 ml of phosphate buffer was then added with 1 ml of cell concentrate (*ca.* 10^8 cells/ml), and the mixture was stirred for approximately one additional minute. The reaction vessel was kept on ice for approximately 2 hours while the polyurethane foam hardened. The foam was removed from the vessel and cut into small pellets (1-3 mm) with a blender. The PU-immobilized cells were rinsed three times with phosphate buffer during cutting of the foam, and the finished PU-immobilized cells were used for the experiments.

To prepare various types of PU co-immobilized cells, various additives were incorporated into the polyurethane prepolymer with the first 1.5 ml of phosphate buffer. For co-immobilizing cells and an adsorbent, powdered activated carbon (0.05 g) or diatomaceous earth (2.0 g) was immobilized with cells. Other adsorbents which could be co-immobilized with the cells in PU would be recognized by those persons of ordinary skill in the art.

To prepare the co-immobilized cells and slow-release formulations of nitrogen and phosphorus, soybean lecithin (0.2 g), phenylacetylurea (0.2 g), and diatomaceous earth (2.0 g) were used. When these slow-release formulations of nutrients were co-immobilized, distilled water, instead of phosphate buffer, was used to rinse the formed co-immobilized cells in order to reduce the possible interference from the phosphate buffer. Other sources could also be used, as would be readily accepted in the art.

To control the density of PU-immobilized cells, several formulations of densification agents were co-immobilized with cells, respectively. These formulations included (1) 4.0 g silica (SiO_2); (2) 2 g SiO_2 and 1 g diatomaceous earth; (3) 2 g aluminum (Al_2O_3) and 1 g diatomaceous earth; and (4) 2 g Al_2O_3 and 2 g SiO_2.

Determination of cell viability. To determine the cell viability of biocomposite materials, PVA capsules or vermiculite carriers were added into 50 ml of phosphate

buffer (0.025 M). This mixture was shaken at 150 rpm and 30RC for 30 minutes (PVA capsules were completely dissolved within 30 minutes). The mixture was diluted to the required concentrations and plated on LB agar plates. The agar plates were incubated at 30RC for 2 to 3 days before counting. Viability of non-encapsulated cells was determined in the same procedure with the dissolution process omitted. Since the polyurethane polymer is not water-soluble, the viability of PU-immobilized cells could not be determined by this procedure.

Mineralization of ^{14}C-labeled compounds in non-contaminated soil. Soil from Southern Illinois University (no history of exposure to PAHs; herein referred to as SIU soil) was used in the mineralization experiments with PVA-encapsulated and vermiculite-carried PAH-degrading microorganisms. In the soil slurry experiments, soil (1.5 g) was added into biometer flasks (300 ml, Bellco) and autoclaved at 121RC twice (each for 30 minutes). Mixed ^{14}C-labeled and unlabeled phenanthrene or fluoranthene in acetone (0.1 ml, 100 mg/ml), with radioactivity of approximately 30,000 DPM, was added to the soil in each biometer flask, and the acetone was allowed to evaporate. Fifty milliliters of MS (II) medium (Mueller, J.G., P.J. Chapman, P.H. Pritchard [1989] *Appl. Environ. Microbiol.* 55:3085-3090), together with 0.015 ml of Triton X-100, was added to each flask. Finally, PVA-encapsulated, vermiculite-carried, or free cells, each with a known bacterial number, were added into each flask to a desired inoculum concentration. One milliliter of 2 N NaOH solution was placed in the side arm of the flask to trap the $^{14}CO_2$ produced.

In the soil slurry experiments, the flasks were shaken at 150 rpm and 30RC. At the intervals indicated, the NaOH solution was removed for determining $^{14}CO_2$ produced and replaced with the fresh trapping solution. The used NaOH solution was mixed with 15 ml of scintillation cocktail (Ultimate Gold), and the radioactivity of this mixture was determined by a liquid scintillation counter (Packard). Data of $^{14}CO_2$ release were corrected for background radioactivity and for counting efficiency. The latter was determined with an internal standard of ^{14}C-toluene. Samples of the soil slurry were also taken for cell counts.

For use with moist soil (solid-phase application), 40 g of sterile soil (autoclaved twice at 121RC) was placed into each biometer flask. After adding the mixed ^{14}C-labeled and unlabeled phenanthrene or fluoranthene, 2 ml of 10X

concentrated MS (II) medium and 0.015 ml of Triton X-100 were added into the soil. The soil was then inoculated with PVA-encapsulated, vermiculite-carried, or free cells plus 6 ml of sterile deionized water (to a final soil moisture content of 22%). The cell number of each inoculant preparation was determined before the inoculation (see Determination of cell viability, *supra*), and a desired amount of each inoculant material was used for inoculation. In addition, PVA-encapsulated cells were also used in the soil in a non-predissolved form. All the soil samples were manually mixed before incubation. The soil samples were incubated at 30RC under a stationary condition. The procedure determining the $^{14}CO_2$ produced was the same as described above.

PU-immobilized cells were tested in MS (II) medium (with no soil), since the preferred application of these biocomposites is with aqueous phase treatment. The prepared PU-immobilized cells of strain EPA 505 (see above) were divided into two equal parts (by weight), and one part of them added into 50 ml of MS (II) medium containing 20 mg of mixed ^{14}C-labeled and unlabeled fluoranthene. Other procedures were the same as described above for mineralization experiments with soil slurry. Data reported on the mineralization experiments are the average of duplicate samples.

Degradation in creosote-contaminated soil. A creosote-contaminated soil from Nashua, NH was used. For the soil slurry experiments, 100 ml of MS (II) medium and 10 g of the field soil were added into a 300 ml biometer flask. Triton X-100 was added to a final concentration of 0.015% (v/v). The soil slurry was inoculated with PVA-encapsulated, vermiculite-carried, or non-encapsulated cells of strain CRE 7 to obtain a desired inoculum concentration. Slurry samples using only indigenous organisms (with no inoculation) and heat-killed control (sterile soil without inoculation) were also incubated. At every 3 to 4 days, slurry samples (5 ml) were removed for determining concentrations of creosote constituents by GC analysis (Mueller *et al.* [1990], *supra*). Slurry samples were also removed for cell counts as described above.

In the experiments with moist soil (solid-state remediation), 40 g of the creosote-contaminated field soil were added into a 300 ml biometer flask. Two milliliters of 10X concentrated MS (II) medium, 6 ml of sterile deionized water, and 0.015 ml of Triton X-100 were added into the soil. The soil was inoculated with PVA-encapsulated, vermiculite-carried, or non-encapsulated cells of strain CRE 7 to

a desired inoculum concentration. Four control experiments were also conducted: (1) soil samples using only indigenous organisms (without any inoculation); (2) soil samples using sterile PVA capsules (with no cells encapsulated); (3) soil samples using sterile vermiculite (containing LB broth and no cells); and (4) sterile soil without inoculation (heat-killed control). All the soil samples were manually mixed before incubation. During the degradation experiment, CO_2 produced from the soil samples was trapped by 1 ml of 2 N NaOH solution placed in the side arm of the biometer flask. At the indicated intervals, the formed sodium carbonate was titrated using standard H_2SO_4 solution and thus CO_2 produced calculated. At day 20, approximately 5 g of the soil sample was removed from each flask for determining the concentrations of creosote constituents by GC analysis (Mueller *et al.* [YEAR?] *supra*). Data reported on the degradation in the creosote-contaminated soil under slurry and solid-phase conditions are the average of duplicate samples.

The specific mineralization rates based on per unit inoculant for both strains CRE 7 and EPA 505 are shown in Tables 2, 3, 6, and 7. The specific mineralization rates are based on per unit inoculant, which compare the degrading activity of PVA-encapsulated/vermiculite-carried inoculants with non-encapsulated microorganisms.

The PVA-encapsulated or vermiculite-carried inoculants function as well as the fresh, non-encapsulated cells, which were used as a positive control, in soil slurry and soil solid-phase systems. Non-encapsulated inoculants can only maintain their viability for a short period of time, normally from about 10-30 days. However, microbial contamination in the non-encapsulated inoculant preparation can be a serious problem. Therefore, PVA-encapsulated or vermiculite-carried inoculants provide a successful approach to maintaining the viability of microorganisms, which the non-encapsulated inoculants cannot achieve.

Following are examples which illustrate procedures, including the best mode, for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

Example 1 — PVA-Encapsulated Microorganisms

A. Effect of PVA encapsulation process on cell viability. The diameter of the PVA capsules ranges from about 0.8 to 3 mm, and can be regulated during the preparation process. To examine the effect of PVA encapsulation (including freeze drying) on the cell viability, cell counts were performed for PVA-encapsulated strains CRE 7 and EPA 505 before and after encapsulation. With different initial cell concentrations of either strain, the preparation process of PVA capsules resulted in less than 1 log reduction in the cell viability (Table 1). Thus, PVA encapsulation process could maintain a much higher cell viability than the alginate technique. Also, a very high cell density (10^{10} to 10^{11}) can be obtained with per gram of dry PVA capsules, provided that a high concentration of cells was used for encapsulation. Thus, relatively low handling cost for a given amount of inoculants might be expected from the PVA encapsulation technique.

Table 2 shows the effects of encapsulating the nutrients 0.1 ml concentration of inorganic nutrient on the viability of PVA-encapsulated *A. eutrophus* AEO106 (pR0101) cells. The microorganisms were encapsulated in a matrix which comprises 8% PVA, 1.6% kaolin, 0.8% dextrose, and 0.5 ml cell suspension in a total of 2.5 ml.

Table 3 shows the effects of encapsulating the additives 25% PEG600, 10% skim milk, 5% fish protein, 5% yeast extract, 5% Bio2, or 5xLB broth on the viability of encapsulated *A. eutrophus* AEO 106 (pR0101) cells. The microorganisms were encapsulated in a matrix which contained 8% PVA, 1.6% kaolin, 0.8% dextrose, and 0.5 ml cell suspension in a total of 2.5 ml. Inoculant suspension before encapsulation contained $(6.0\pm0.42)\times10^{11}$ CFU/ml.

Table 1. Effects of the PVA encapsulation process on viability of microorganisms[a]

Microorganism	*Pseudomonas* sp. CRE 7		*Pseudomonas paucimobilis* EPA 505	
	Trial 1	Trial 2	Trial 1	Trial 2
Cell count before encapsulation (CFU/ml added cell suspension)	$(4.1\pm0.9) \times 10^{9}$	$(2.2\pm1.41) \times 10^{11}$	$(6.0\pm1.41) \times 10^{9}$	$(8.2\pm5.03) \times 10^{11}$
Cell count immediately after encapsulation (CFU/ml added cell suspension)	$(2.8\pm0.31) \times 10^{10}$	$(3.4\pm0.31) \times 10^{10}$	$(4.2\pm0.49) \times 10^{8}$	$(2.1\pm0.43) \times 10^{11}$

[a] Per gram of dry beads encapsulated 1.73 ml cell suspension.

Table 2. Effects of Encapsulating Nutrients on Viability of Encapsulated *A. eutrophus* AEO 106 (pRO 101) Cells[a]

Cell count before encapsulation (CFU/ml added cell suspension)	Cell count immediately after encapsulation (CFU/ml added cell suspension)		
	Encapsulated cells without nutrient	Encapsulated cells and 0.1 ml concentrate of inorganic nutrient	Encapsulated cells and 0.1 ml 5% yeast extract solution
$(2.8 \pm 0.33) \times 10^{11}$	$(9.7 \pm 0.92) \times 10^{9}$	$(2.0 \pm 0.95) \times 10^{5}$	$(10.1 \pm 1.41) \times 10^{9}$

a. Per gram of dry beads encapsulated 1.73 ml cell suspension.

Table 3. Effects of Encapsulating Additives on Viability of Encapsulated *A. eutrophus* AEO 106 (pRO 101) Cells[a]

Additive	No additive (control)	25 ul PEG 600	0.5 ml 10% skim milk	0.2 ml 5% fish protein
Cell count immediately after encapsulation (CFU/ml added cell suspension)	$(3.1 \pm 0.29) \times 10^9$	$(9.6 \pm 0.38) \times 10^8$	$(1.2 \pm 0.12) \times 10^{10}$	$(1.0 \pm 0.05) \times 10^{10}$

Additive	0.2 ml %5 yeast extract	0.2 ml 5% Bio2	0.2 ml LB broth (5X)
Cell count immediately after encapsulation (CFU/ml added cell suspension)	$(6.4 \pm 0.95) \times 10^9$	$(8.7 \pm 0.68) \times 10^9$	$(2.3 \pm 0.44) \times 10^9$

a. Per gram of dry beads encapsulated 1.73 ml cell suspension.

B. Storage property of PVA-encapsulated microorganisms. To examine the storage life of PVA-encapsulated cells, the dry capsules were stored at 4RC and their viability determined over a 62-day period. For both microorganisms, viability of the capsule products for a storage period of 2 months at 4RC decreased by less than 2 log. In contrast, viability of the non-encapsulated inoculants (free cell concentrate) stored at the same temperature decreased by more than 7 log within 30 days for strain CRE 7 and within 8 days for strain EPA 505. The initial reduction rate in the viability of the encapsulated strain EPA 505 was higher than that of the encapsulated strain CRE 7; however, both reached a similar value at the end of 2 months. These data demonstrate that the PVA encapsulation is a practical means of storage of inoculants.

C. Mineralization of ^{14}C-labeled compounds by PVA-encapsulated microorganisms. Use of ^{14}C-labeled compounds in a sterile soil provided defined conditions to test the degrading activity of encapsulated microorganisms. With this test system, the PAH mineralization activity of encapsulated cells was compared with that of fresh non-encapsulated cells used a positive control.

PVA-encapsulated strain CRE 7 or EPA 505 was first tested in the soil slurry phase spiked with ^{14}C-labeled phenanthrene or fluoranthene. To increase the solubility of the PAH compounds, a surfactant, Triton X-100, was also used. When the PVA-encapsulated cells were used in soil slurry, the PVA capsules completely dissolved within 30 minutes. The mineralization of phenanthrene by the added encapsulated cells of strain CRE 7 was essentially complete within 33 hrs (Figure 1). The fluoranthene mineralization by encapsulated strain EPA 505 is shown in Figure 2. In both cases, the mineralization profiles by PVA-encapsulated cells and non-encapsulated cells exhibited similar trends, and their differences were within the experimental deviation (less than 10%). Thus, PVA encapsulation did not significantly decrease the mineralization activity of these strains.

In the soil solid-phase experiments, PVA-encapsulated strain CRE 7 (Figure 3) or EPA 505 (Figure 4) was added into the moist soil in two ways: (1) PVA capsules were dissolved with water prior to the addition; and (2) PVA capsules were used without predissolution. As was seen in the slurry-phase studies, there was no significant difference in the degradation profiles using the pre-dissolved PVA capsules and the non-encapsulated cells of either strain. When the non-predissolved capsules

were used as inoculants in the soil, no obvious dissolution of the PVA capsules was observed. The commencement of the biodegradation by the non-predissolved capsules was delayed. This effect was most apparent with non-predissolved encapsulated strain EPA 505; here, fluoranthene degradation initiated after 100 hours of incubation. When non-predissolved capsules containing either bacterium were used, the initial bacterial concentration in the soil free of capsules was 50 to 100 times lower than in the sample using the predissolved capsules. These results indicated that the delayed biodegradation was due to the lower number of catalytic units that had been released from the capsules. Thus, increased release of the encapsulated cells can result in enhanced degradation. The phenomenon of slow-release was observed.

The data from these mineralization experiments using PVA-encapsulated and non-encapsulated strains CRE 7 and EPA 505 in relation with the inoculant concentration are summarized in Tables 4 and 5, respectively. The specific mineralization rate in the soil slurry was generally about 10 times higher than in the soil solid-phase for both microbial strains. Since not all the encapsulated cells were released to the soil when non-predissolved capsules were used in the solid-state studies, the actual specific mineralization rates by the bacteria released from the capsules in this case would be higher than the values shown in Tables 4 and 5.

Table 4. Mineralization capacity of non-encapsulated and PVA-encapsulated strain CRE 7[a]

Preparation of inoculant	Inoculant concentration (10^7 CFU/ml or g)	Mineralization rate[b] (µg phen./ml or g-hour)	Specific mineralization rate based on per unit inoculant (µg phen./hour-10^{10} CFU)
In soil slurry			
Free cells	9.2±0.01	3.6±0.20	400±20
Encapsulated cells	1.6±0.00	2.8±0.20	1840±340
In soil			
Free cells	11.3	0.75±0.00	66±22
Encapsulated cells			
Pre-diss.	24.0	1.25±0.20	40±10
As beads	5.0	0.75±0.00	150±0

[a]Values are mean±deviation.
[b]Values were obtained by regression of the data of phenanthrene mineralization in the linear region.

Table 5. Mineralization capacity of non-encapsulated and PVA-encapsulated strain EPA 505[a]

Preparation of inoculant	Inoculant concentration (10^7 CFU/ml or g)	Mineralization rate[b] (µg fluo./ml or g-hour)	Specific mineralization rate based on per unit inoculant (µg fluo./hour-10^{10} CFU)
In soil slurry			
Free cells	11.8±0.03	2.6±0.26	218±22
Encapsulated cells	2.1±0.68	2.6±0.08	1219±38
In soil			
Free cells	14.8±0.04	0.59±0.00	40±0
Encapsulated cells			
Pre-diss.	2.6±0.85	0.46±0.12	174±45
As beads	2.6±0.85	0.10±0.05	38±19

[a]Values are mean±deviation.
[b]Values were obtained by regression of the data of fluoranthene mineralization in the linear region.

D. Degradation by PVA-encapsulated microorganisms in creosote-contaminated soil. The utility of PVA-encapsulated was shown for remediating a creosote-contaminated soil in a soil slurry reactor and a solid-state process at the bench-scale. Since strain CRE 7 is a phenanthrene degrader, the primary measurement in these experiments was the degradation of phenanthrene, although removal of other creosote constituents in the soil was also analyzed chemically.

When PVA capsules (14-day storage at 4RC) were used in a soil slurry reactor containing the creosote-contaminated soil, approximately 90% of phenanthrene was degraded after 24 days (Table 6). The use of fresh, non-encapsulated inoculum gained the similar extent of biodegradation. When no inoculum was used, biodegradation of phenanthrene in the samples using indigenous organisms was not observed. With inoculation by both PVA-encapsulated and non-encapsulated cells, most of 2 to 3-ring creosote constituents were biodegraded.

Table 6. Effect of PVA-encapsulation on the activity of strain CRE 7 in a slurry reactor containing a creosote-contaminated soil (Nashua, NH)

Inoculum preparation	PVA-encaps. (14-day storage)	Free cells (Fresh)	No inoculum
Ini. inoculum conc. (CFU/ml)	2.9×10^5	0.8×10^5	0
% phenanthrene biodegraded at day 24*	89.8±1.4	89.9±10.3	0±0

* Data are the average of duplicate treatment samples after 24-day bioreactor operation. The initial phenanthrene concentrations varied with the soil samples.

After PVA-encapsulated or non-encapsulated strain CRE 7 was added into the creosote-contaminated soil amended with inorganic nutrients and Triton X-100, the microbial activity, as measured by CO_2 generation, was determined under a solid-phase condition. The microbial activity was higher in the sample using PVA-encapsulated strain CRE 7 than using non-encapsulated cells. With no inoculation, significant CO_2

production was only seen after 10 days of incubation. The same CO_2 generation profile was also determined from another control sample that used sterile PVA capsules. No CO_2 was produced from the sterile soil (heat-killed control).

Table 5 shows the degradation of phenanthrene by PVA-encapsulated and non-encapsulated strain CRE 7 and indigenous organisms in the solid-state remediation experiment. At day 20, approximately 60% of phenanthrene in the soil was degraded by PVA-encapsulated, and 40% by non-encapsulated strain CRE 7. For the samples with no inoculation, the degradation results were inconsistent, as indicated by the large standard deviation value (Table 7). These results demonstrate the effectiveness of PVA-encapsulated inoculants in bioremediation of creosote-contaminated soils.

Table 7. Effect of PVA-encapsulation on the activity of strain CRE 7 in solid-state remediation of a creosote-contaminated soil (Nashua, NH)

Inoculum preparation	PVA-encaps. (14 day storage)	Free cells (Fresh)	No inoculum
Ini. inoculum conc. (CFU/g)	1.7×10^8	1.5×10^9	0
% phenanthrene biodegraded*	59.0±4.9	41.8±9.8	34.8±43.3

*Data are the average of duplicate treatment samples at day 20.

Example 2 — Vermiculite-Carried Microorganisms

A. Growth and storage property of vermiculite-carried microorganisms. The moisture content of the vermiculite after inoculation was approximately 200% (w/w). Under this moisture condition, the vermiculite powder absorbed almost all the water added. This wet vermiculite powder served as a solid-state fermentation matrix. Vermiculite-carried strains CRE 7 and EPA 505 were shown to be viable for more than 60 days during incubation and storage at room temperature. Cell number of both vermiculite-carried strains CRE 7 and EPA 505 increased by approximately 10^4 times in the first week, indicating a growth phase for the inoculants on the vermiculite.

During continued storage for about 2 months at room temperature, viability of vermiculite-carried strain CRE 7 decreased by less than 1 log. Under the same storage condition, viability of vermiculite-carried strain EPA 505 reduced by about 3 log. Thus, vermiculite can provide a suitable matrix for cell growth, and the CRE 7 and EPA 505 vermiculite-carried microbial strains can be successfully stored for more than two months without total loss of viability.

B. Mineralization of ^{14}C-labeled compounds by vermiculite-carried microorganisms. When vermiculite-carried cells (after 7-day storage at room temperature) were added into the soil slurry, they were uniformly distributed in the slurry. The initial mineralization profile by the vermiculite-carried strain CRE 7 (Figure 5) or EPA 505 (Figure 6) was similar to that by the fresh, non-immobilized cells. In the case using strain CRE 7, the extent of phenanthrene mineralization by the vermiculite-carried inoculant appeared to be lower (about 20%) than that using the fresh, non-immobilized cells, suggesting the possible effect of adsorption of phenanthrene by vermiculite particles on the bioavailability (Figure 5). However, this effect was not significant when using strain EPA 505 (Figure 6). Here, the difference in the degradation profiles by free and vermiculite-carried strain EPA 505 was within the range of experimental deviation (less than 10%).

In the solid-phase soil experiments using vermiculite-carried strain CRE 7 (Figure 7) or EPA 505 (Figure 8) (after 7-day storage), no significant difference was seen in the initial degradation rates by the vermiculite-carried and the fresh, non-immobilized cells.

The data from these mineralization experiments using vermiculite-carried and non-immobilized strains CRE 7 and EPA 505 in relation with the inoculant concentration are summarized in Tables 8 and 9, respectively. In either case of soil slurry or soil solid-phase, the specific mineralization rate by vermiculite-carried and free cells was similar. The specific rate in soil slurry was approximately 10 times higher than in the soil solid-phase.

Table 8. Mineralization capacity of non-immobilized and vermiculite-carried strain CRE 7[a]

Preparation of inoculant	Inoculant concentration (10^7 CFU/ml or g)	Mineralization rate[b] (μg phen./ml or g-hr)	Specific mineralization rate based on per unit inoculant (μg phen./hr-10^{10} CFU)
In soil slurry			
Free cells	9.2±0.01	3.6±0.20	400±20
Ver. cells	6.3±0.00	2.4±0.80	420±160
In soil			
Free cells	43.0±2.10	0.8±0.00	20±0
Ver. cells	81.0±0.00	1.0±0.00	12±0

[a]Values are mean±deviation.
[b]Values were obtained by regression of the data of phenanthrene mineralization in the linear region.

Table 9. Mineralization capacity of non-immobilized and vermiculite-carried strain EPA 505[a]

Preparation of inoculant	Inoculant concentration (10^7 CFU/ml or g)	Mineralization rate[b] (μg fluo./ml or g-hr)	Specific mineralization rate based on per unit inoculant (μg fluo./hr-10^{10} CFU)
In soil slurry			
Free cells	11.8±0.03	2.6±0.26	218±22
Ver. cells	8.8±0.41	2.3±0.24	258±27
In soil			
Free cells	14.7±0.04	0.6±0.00	40±0
Ver. cells	11.0±0.51	0.5±0.00	47±0

[a]Values are mean±deviation.
[b]Values were obtained by regression of the data of fluoranthene mineralization in the linear region.

C. Degradation by vermiculite-carried cells in creosote-contaminated soil. When the vermiculite-carried strain CRE 7 after 14-day storage was used in a soil slurry reactor containing the creosote-contaminated soil, approximately 90% of phenanthrene was degraded after 24 days (Table 10). Similarly, inoculation with fresh, non-immobilized cells reached the similar percentage of biodegradation. Without inoculation, no biodegradation of phenanthrene was found.

Table 10. Effect of vermiculite formulation on the activity of strain CRE 7 in a slurry reactor containing a creosote-contaminated soil (Nashua, NH)*

Inoculum preparation	Ver. inoc. (14-day storage	Free cells (Fresh)	No inoculum
Ini. inoculum	$1.1x10^5$	$0.8x10^5$	0
% phenanthrene biodegraded at day 24*	90.7±5.43	89.9±10.3	0±0

*Data are the average of duplicate treatment samples after 24-day bioreactor operation. The initial phenanthrene concentrations varied with the soil samples.

After vermiculite-carried and non-immobilized strain CRE 7 were added to the creosote-contaminated soil amended with inorganic nutrients and Triton X-100, the microbial activity (CO_2 generation) under solid-phase conditions was determined. Microbial activity in the soil using vermiculite-carried cells was the highest of the treatments tested. Without inoculation, significant CO_2 production was only observed after 10 days of incubation. The same CO_2 production profile was seen from another control sample using sterile vermiculite. No CO_2 was produced from the sterile soil (heat-killed control). Table 11 shows the degradation of phenanthrene by the vermiculite-carried inoculant, non-immobilized inoculant, and indigenous organisms in the solid-state remediation. At day 20, approximately 50% of phenanthrene in the soil was degraded by vermiculite-carried, and 40% by non-immobilized strain CRE 7. These results demonstrate the potential effectiveness of vermiculite-carried inoculants in bioremediation of actual contaminated soils.

Table 11. Effect of vermiculite formulation on the activity of strain CRE 7 in solid-state remediation of a creosote-contaminated soil (Nashua, NH)

Inoculum preparation	Ver. inoc. (14-day storage)	Free cell (Fresh)	No inoculum
Ini. inoculum conc. (CFU/g)	$9.7x10^7$	$1.5x10^9$	0
% phenanthrene biodegraded*	51.5±19.6	41.8±9.8	34.8±43.3

*Data are the average of duplicate treatment samples at day 20.

Example 3 — PU-Immobilized Microorganisms

A. Effect of immobilization and co-immobilized adsorbents on the degrading activity. Polyurethane (PU) polymer was used to immobilize *Pseudomonas paucimobilis* EPA 505 (a fluoranthene degrader). Adsorbent and nutrient powder were also immobilized with the microbial cells. One concern for any immobilization process is its effect on the activity of the microorganisms. When polyurethane pellets containing cells of strain EPA 505 were used in MS (II) medium spiked with ^{14}C-labeled fluoranthene, it was observed that the fluoranthene mineralization profiles by PU-immobilized cells was similar to that by non-immobilized cells (Figure 9). Incubation was conducted in a shaken flask containing MSII medium with Triton X-100, 200 mg fluoranthene, and $8X10^6$ CFU/ml of inoculum. Since the same inoculum size was used for the mineralization experiment by the free cells and for the immobilization process, this result showed that the PU immobilization process advantageously does not significantly affect the catabolic activity of this strain.

Use of adsorbents in an immobilization matrix can aid in the rapid removal of toxic compounds from the environment. Two issues need to be addressed when using co-immobilized adsorbents: (1) microbial activity after the co-immobilization with adsorbents; and (2) availability of adsorbed compounds to the immobilized cells. Diatomaceous earth or powdered activated carbon was co-immobilized with strain EPA 505 in a polyurethane matrix. When these co-immobilized cells and adsorbents

were tested in the degradation medium, it was found that: (1) the degrading activity of the cells co-immobilized with adsorbents was indistinguishable from that on non-immobilized cells; and (2) immobilized cells with adsorbents mineralized as much fluoranthene as free cells, indicating that the degradation of the absorbed fluoranthene on the co-immobilized adsorbent particles was complete (Figure 9).

B. Effect of co-immobilized slow-release formulations of nitrogen and phosphorus. Slow-release formulations of nitrogen (phenylacetylurea) and phosphorus (soybean lecithin) were co-immobilized with strain EPA 505 and diatomaceous earth in the polyurethane matrix, and tested in distilled water for fluoranthene biodegradation. As a positive control, immobilized cells with external nitrogen and phosphorus (provided by MS (II) medium) was also used. As shown in Figure 10, within 180 hours of incubation, the cells co-immobilized with slowly released nitrogen and phosphorus could degrade fluoranthene as fast as the immobilized cells using external nitrogen and phosphorus. Beyond 180 hours, the degradation rate by cells co-immobilized with the nutrients decreased and the degradation kinetics became linear. Incubation was conducted in a shaken flask containing distilled water of MS medium with Triton X-100, 20 mg fluoranthene, and $8X10^6$ CFU/ml of inoculum. These results show that the co-immobilized slowly released nitrogen and phosphorus can support the biodegradation.

C. Effect of densification agents. In many applications, such as in treatment systems for marine sediments and fluidized bed reactors, density of immobilized cells is an important parameter for maintaining these biocatalysts at the required spatial location. Therefore, suitable densification agents decrease the tendency of the immobilized cells to float. Three densification agents, silica (SiO_2), aluminum (Al_2O_3), and/or diatomaceous earth, were co-immobilized with strain EPA 505 in the polyurethane matrix. As shown in Figure 11, when silica and/or diatomaceous earth was used, the degrading activity of the co-immobilized cells was similar to that of immobilized cells with no densification agent. These results show that silica and diatomaceous earth are two suitable densification agents. Different amounts of these densification agents can be used in the co-immobilization systems depending on the required density and application principle. These amounts would be readily ascertained by persons of ordinary skill in the art without undue experimentation.

Thus, it was found that the degrading activity of the cells co-immobilized with adsorbents was similar to that of non-immobilized cells and that the use of the immobilized cells with adsorbents gained the similar percentage of mineralization as free cells did, indicating that the degradation of the adsorbed fluoranthene was complete.

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

Claims

1. A biocomposite comprising a microorganism and an additive in a formulation matrix, wherein said microorganism is capable of degrading an organic environmental contaminant.

2. The biocomposite, according to claim 1, wherein said microorganism is selected from the group consisting of *Pseudomonas* spp., *Mycobacterium* spp. and *Alcaligenes* spp.

3. The biocomposite, according to claim 1, wherein said microorganism is *Pseudomonas paucimobilis* strain EPA 505 or strain CRE7.

4. The biocomposite, according to claim 3, wherein said microorganism is *Pseudomonas paucimobilis* EPA 505.

5. The biocomposite, according to claim 1, wherein said additive comprises a nutrient, an adsorbent, or a density agent.

6. The biocomposite, according to claim 5, wherein said additive is a nutrient, said nutrient comprising a source of phosphorous or nitrogen for said microorganism.

7. The biocomposite, according to claim 6, wherein said nutrient is selected from the group consisting of soybean lecithin, phenylacetylurea, skim milk, fish protein, and yeast extract.

8. The biocomposite, according to claim 1, wherein said formulation matrix is an encapsulation or immobilization matrix.

9. The biocomposite, according to claim 1, wherein said formulation matrix is selected from the group consisting of polyvinyl alcohol (PVA), vermiculite, and polyurethane.

10. The biocomposite, according to claim 1, wherein the microorganism-degraded contaminant is a polycyclic aromatic hydrocarbon.

11. The biocomposite, according to claim 1, wherein the microorganism-degraded contaminant is an organic pesticide.

12. The biocomposite, according to claim 1, wherein said additive is a density agent.

13. The biocomposite, according to claim 12, wherein said density agent is silica.

14. A method for bioremediating an organic environmental contaminant in soil, air, or water, wherein said method comprises contacting said contaminated soil, air, or water with a biocomposite, said biocomposite comprising a microorganism and an additive in a formulation matrix, wherein said microorganism is capable of degrading an organic environmental contaminant.

15. The method, according to claim 14, wherein said environmental contaminant is adsorbed onto said biocomposite and is simultaneously degraded.

16. The method, according to claim 14, wherein said contacting step occurs in a groundwater circulation well comprising a bioreactor, said bioreactor having as an inoculant a biocomposite, said biocomposite comprising a microorganism and an additive in a formulation matrix, wherein said microorganism is capable of degrading an organic environmental contaminant.

17. The method, according to claim 14, wherein said environmental contaminant is a high molecular weight polycyclic aromatic hydrocarbon.

18. The method, according to claim 14, wherein said microorganism is *Pseudomonas paucimobilis* EPA 505.

19. A bioreactor, said bioreactor comprising a biocomposite as an inoculant, said biocomposite comprising a microorganism and an additive in a formulation matrix, wherein said microorganism is capable of degrading an organic environmental contaminant.

20. The bioreactor, according to claim 19, wherein said bioreactor is an *in situ* groundwater circulation well.

Figure 1

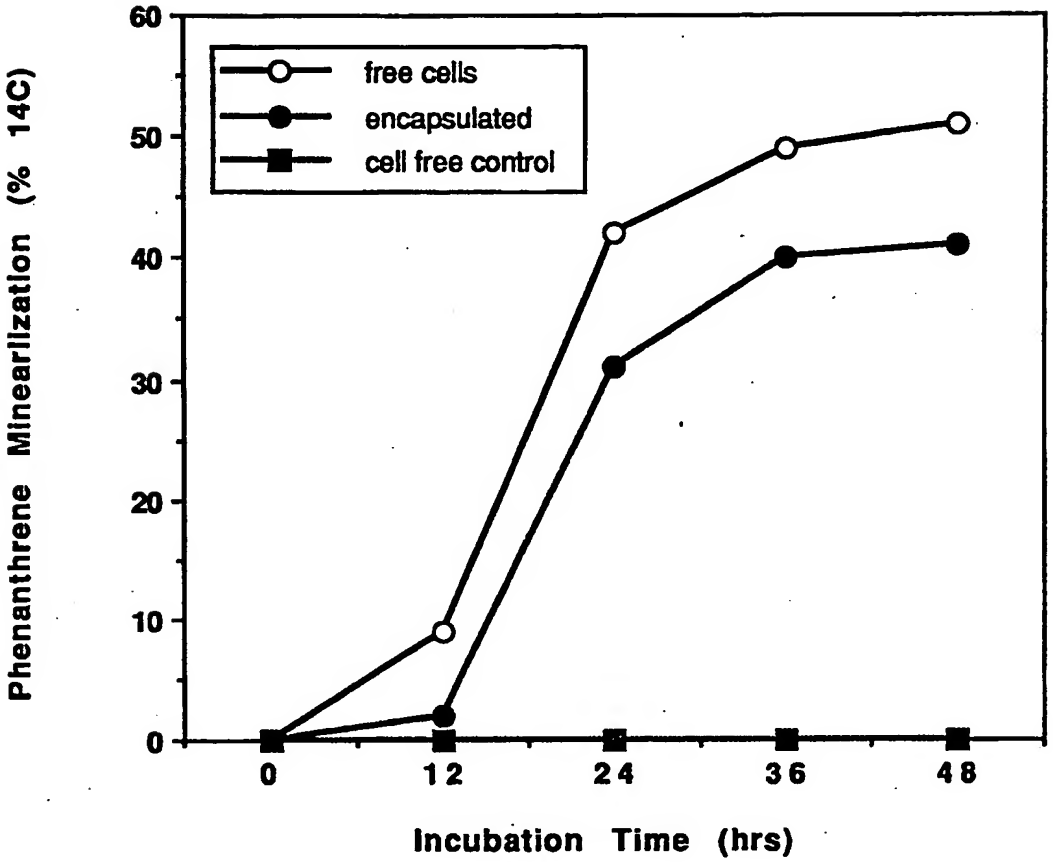
60
50
40
30
20
10
0
free cells
encapsulated
cell free control
Phenanthrene Minearlization (% 14C)
0
12
24
36
48
Incubation Time (hrs)

Figure 2

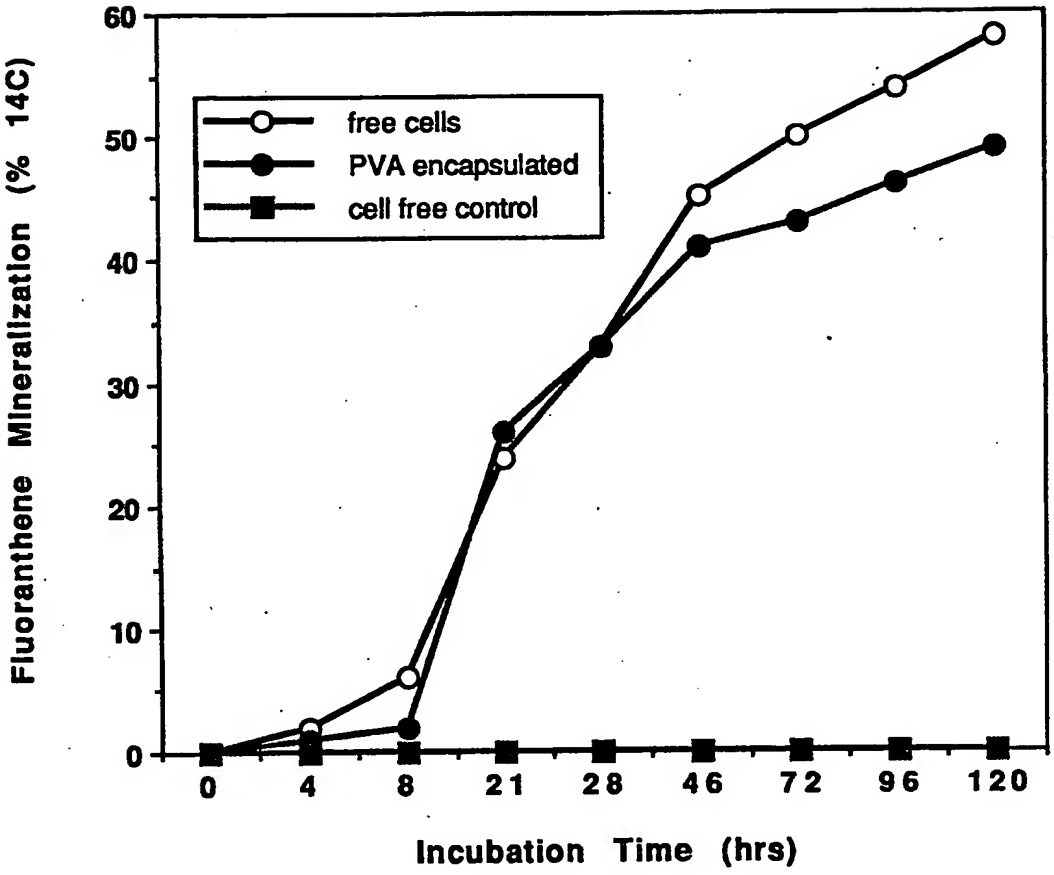
Fluoranthene Mineralization (% 14C)
60
50
40
30
20
10
0
free cells
PVA encapsulated
cell free control
0
4
8
21
28
46
72
96
120
Incubation Time (hrs)

Figure 3

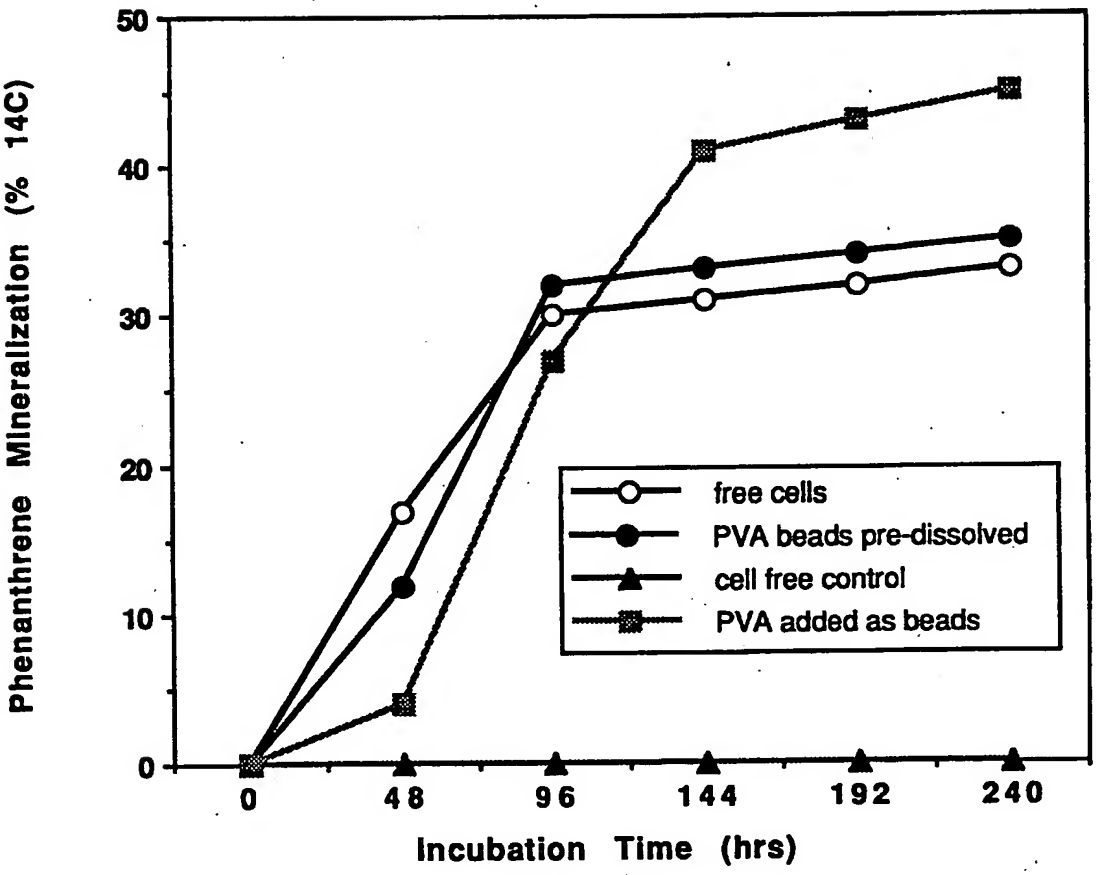
Phenanthrene Mineralization (% 14C)
50
40
30
20
10
0
0
48
96
144
192
240
Incubation Time (hrs)
free cells
PVA beads pre-dissolved
cell free control
PVA added as beads

Figure 4

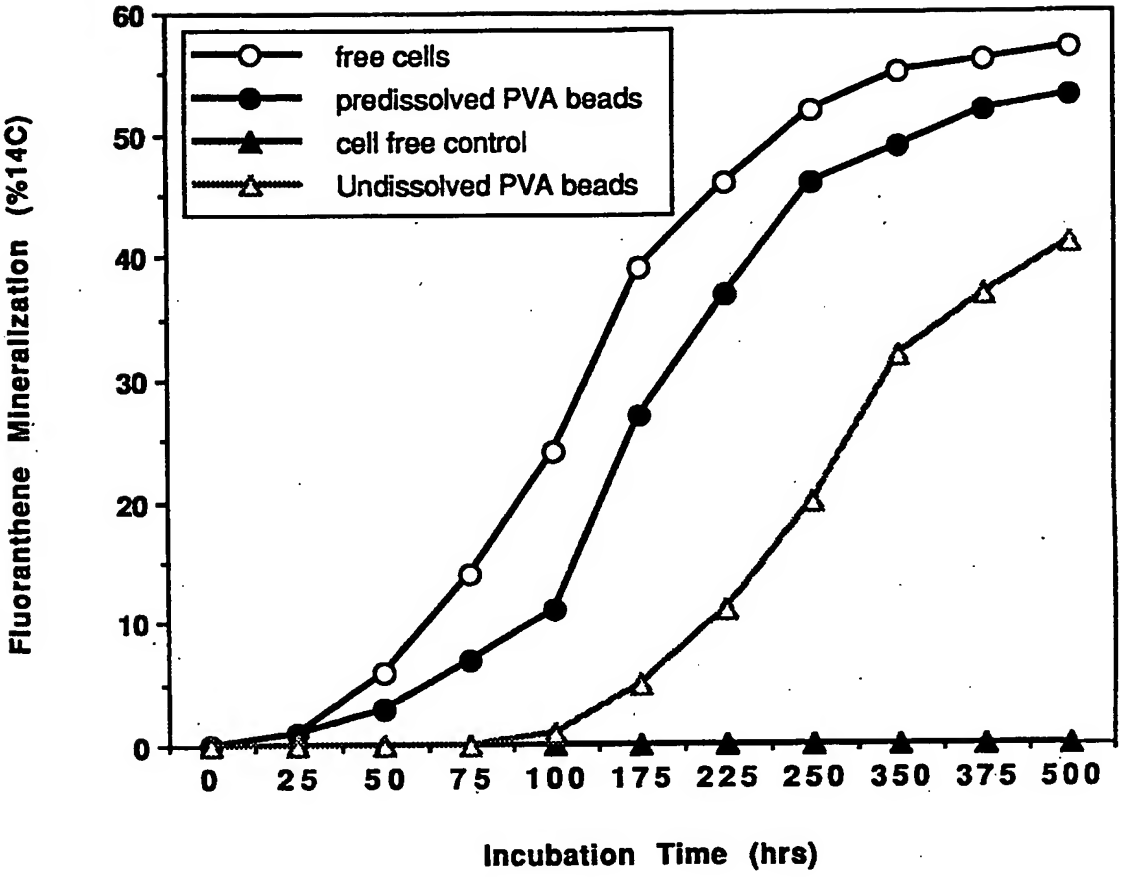

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Figure 5

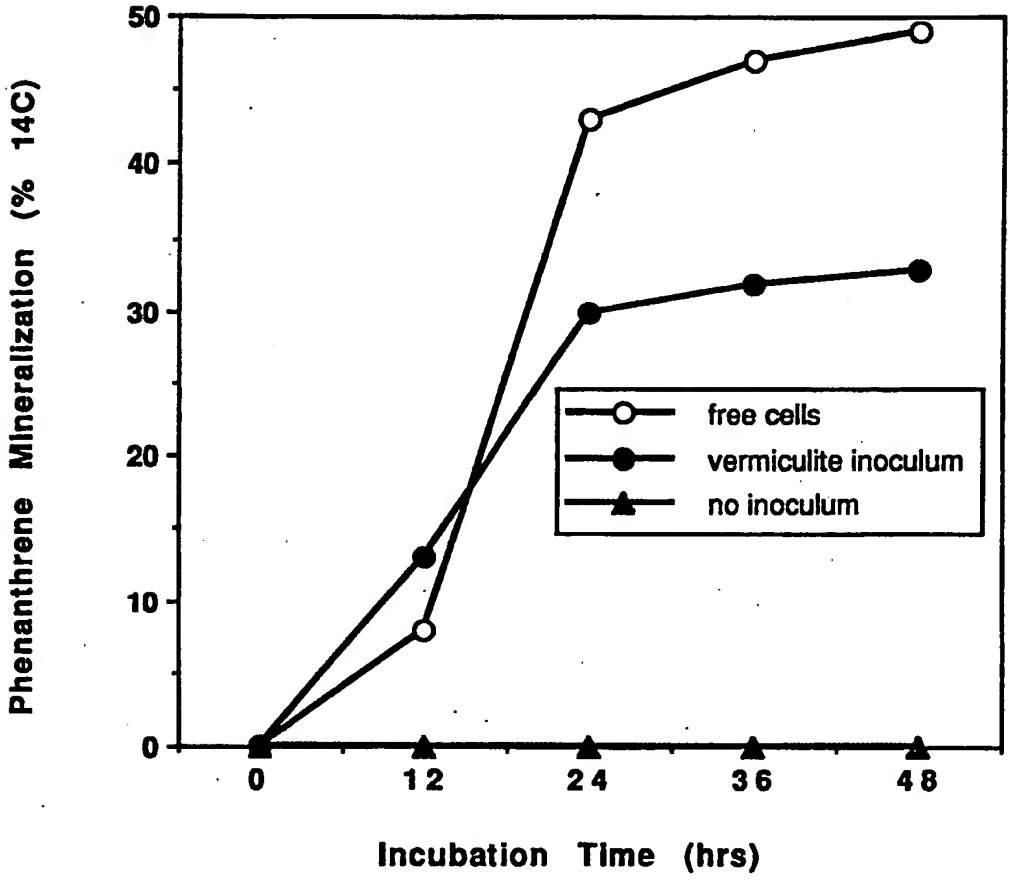

Phenanthrene Mineralization (% 14C)
50
40
30
20
10
0
0
12
24
36
48
Incubation Time (hrs)
free cells
vermiculite inoculum
no inoculum

Figure 6

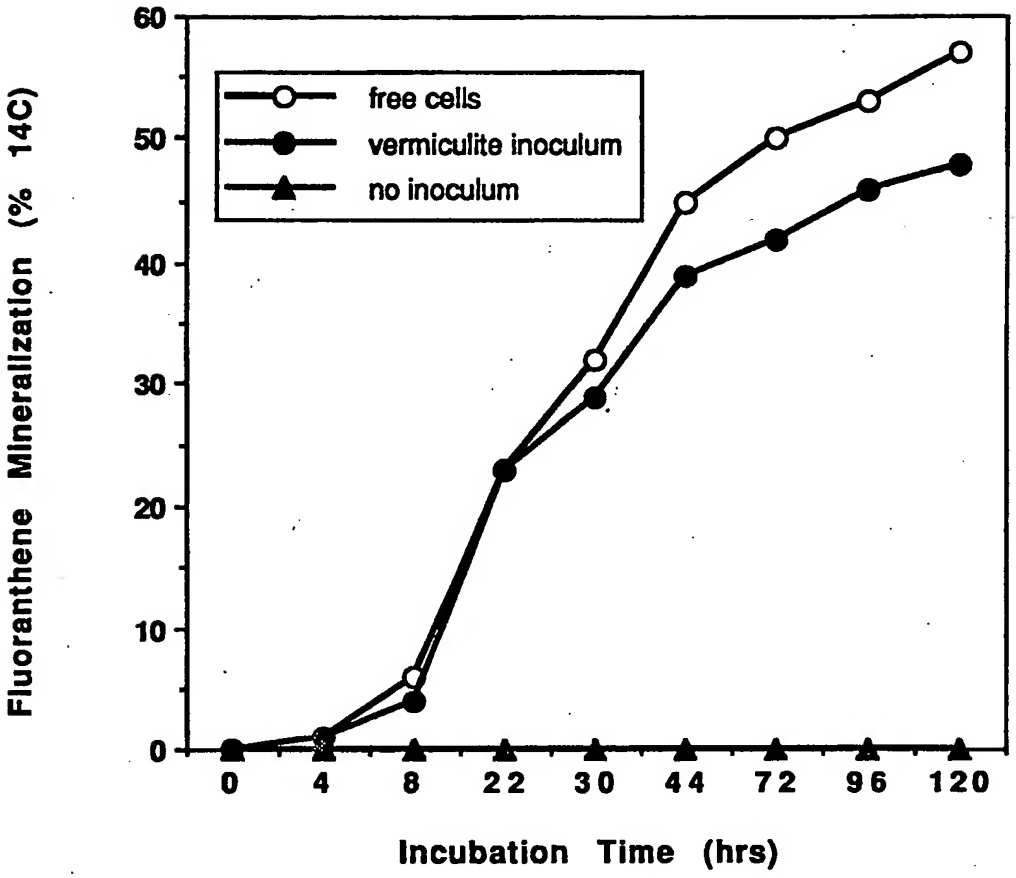

60
50
40
30
20
10
0
free cells
vermiculite inoculum
no inoculum
Fluoranthene Mineralization (% 14C)
0
4
8
22
30
44
72
96
120
Incubation Time (hrs)

Figure 7

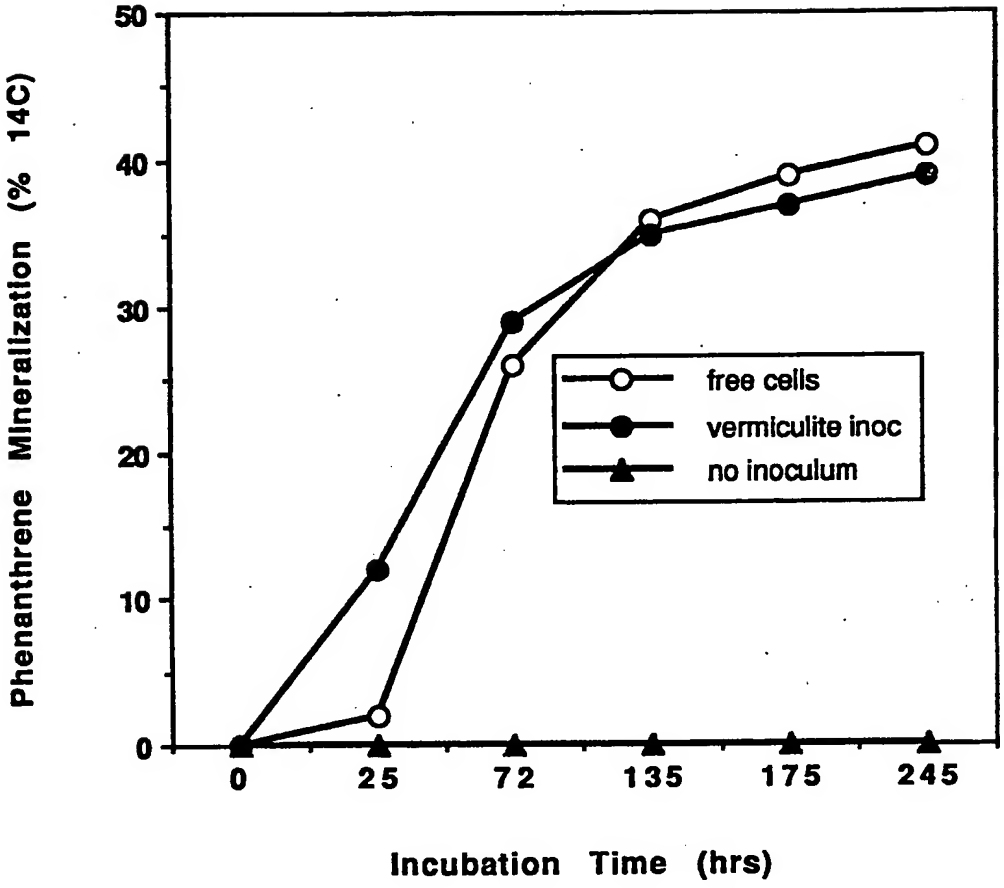

Phenanthrene Mineralization (% 14C)
50
40
30
20
10
0
0
25
72
135
175
245
Incubation Time (hrs)
free cells
vermiculite inoc
no inoculum

Figure 8

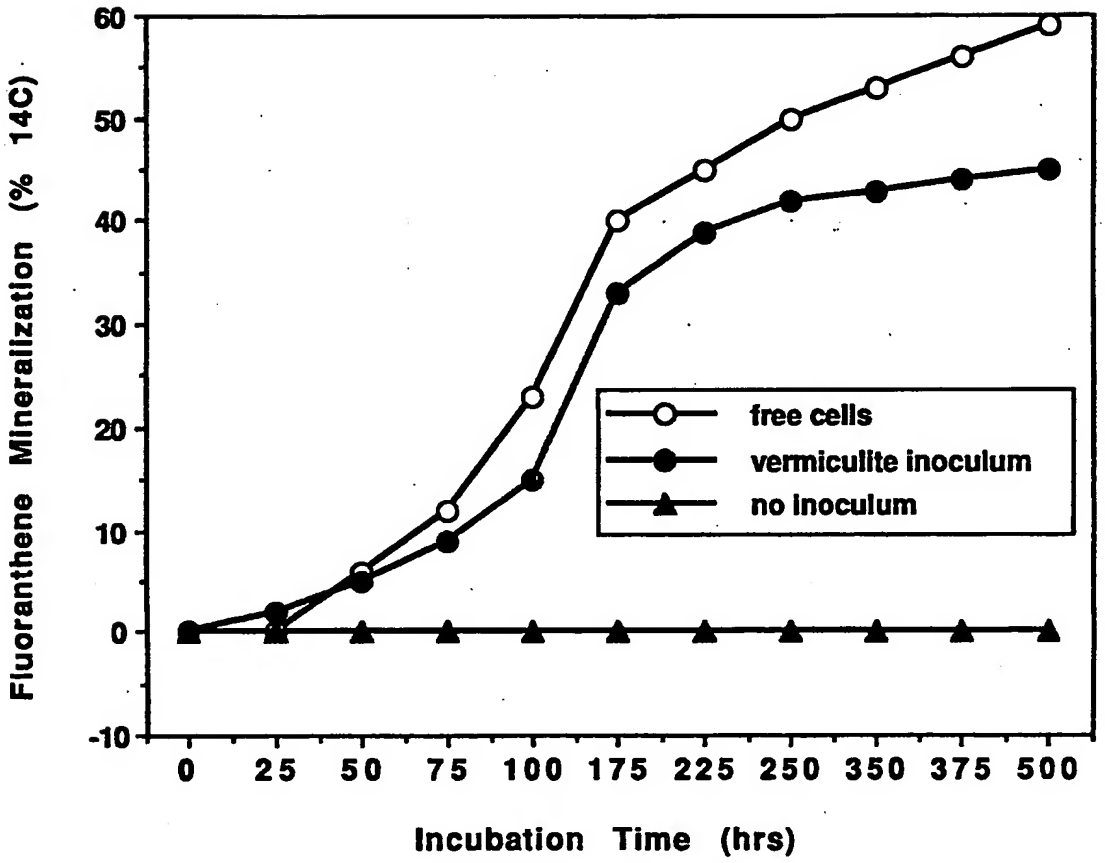
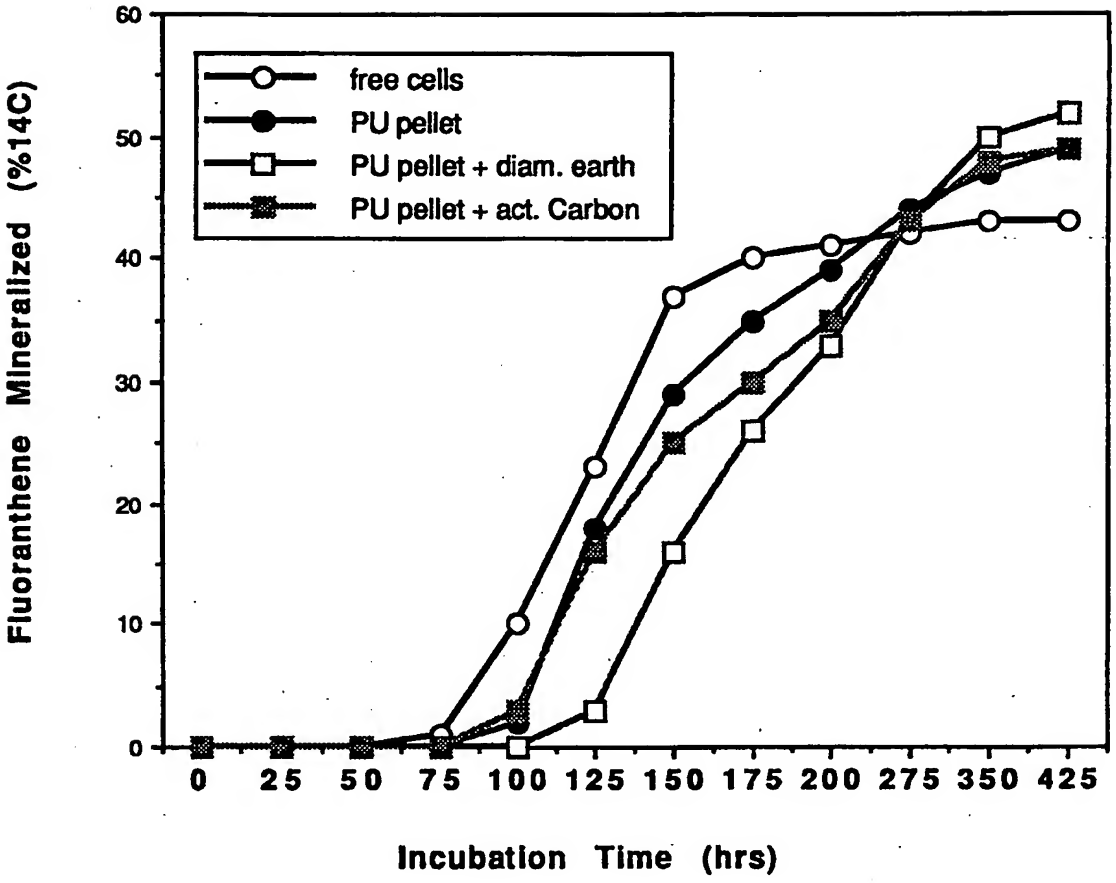

Fluoranthene Mineralization (% 14C)
60
50
40
30
20
10
0
-10
0
25
50
75
100
175
225
250
350
375
500
Incubation Time (hrs)
free cells
vermiculite inoculum
no inoculum

Figure 9



RECTIFIED SHEET (RULE 91)
ISA/EP

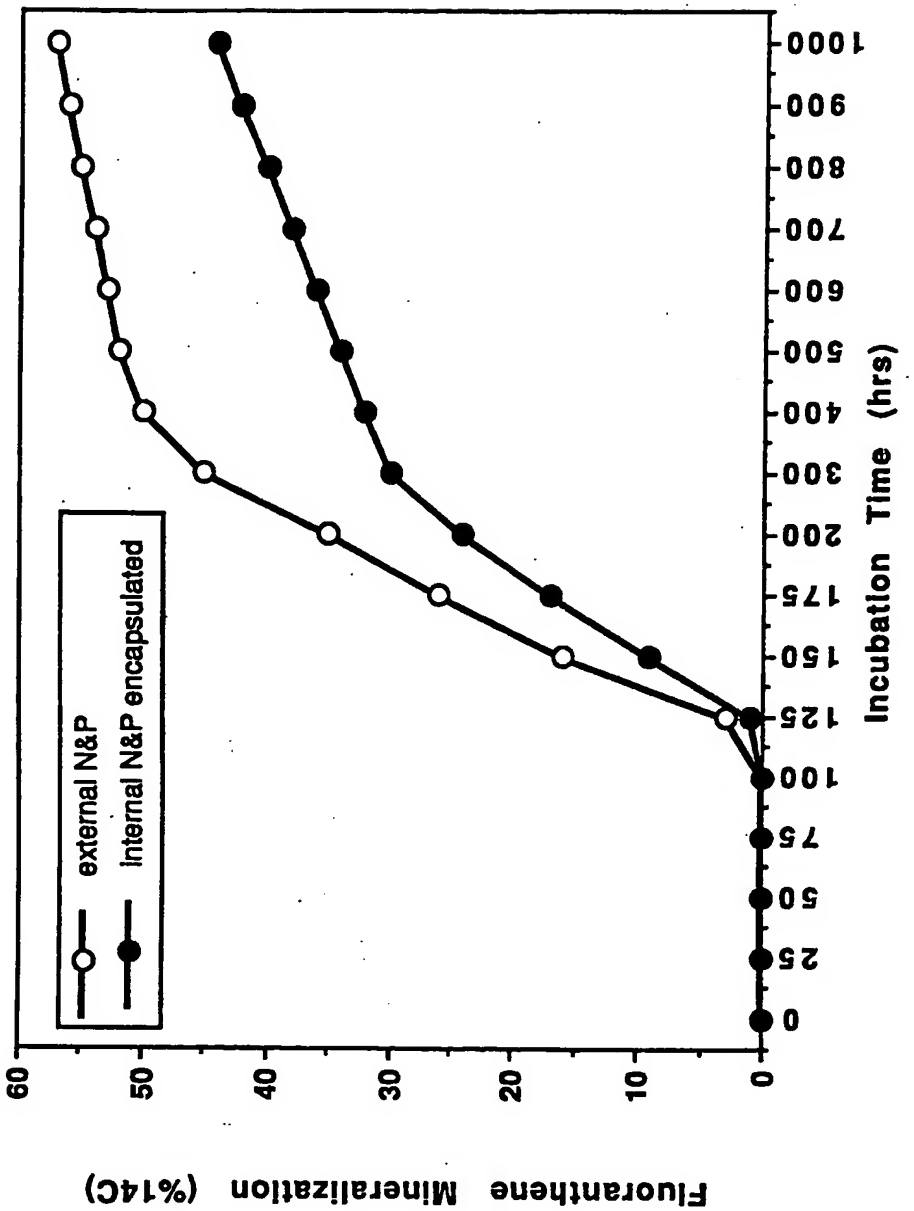
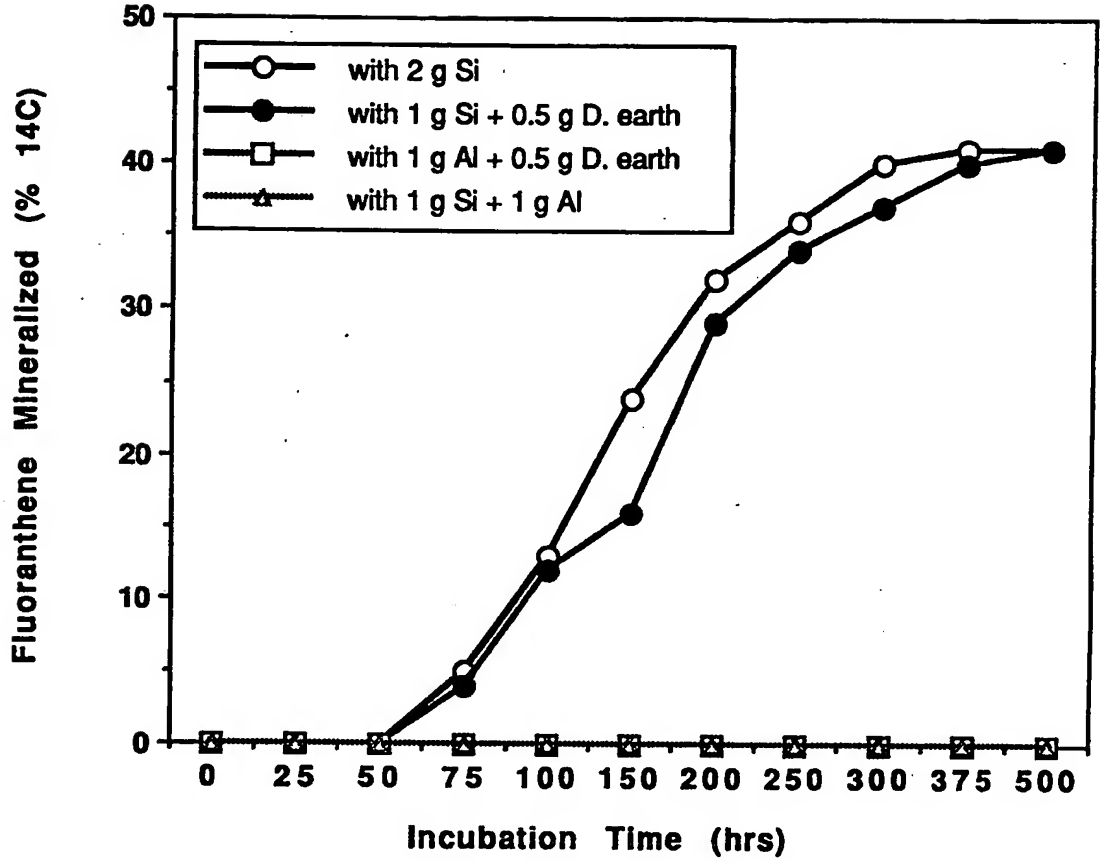

Incubation Time (hrs)
0
25
50
75
100
125
150
175
200
300
400
500
600
700
800
900
1000
external N&P
internal N&P encapsulated
0
10
20
30
40
50
60
Fluoranthene Mineralization (%14C)

Figure 10

Figure 11


with 2 g Si
with 1 g Si + 0.5 g D. earth
with 1 g Al + 0.5 g D. earth
with 1 g Si + 1 g Al
Fluoranthene Mineralized (% 14C)
0
10
20
30
40
50
0 25 50 75 100 150 200 250 300 375 500
Incubation Time (hrs)

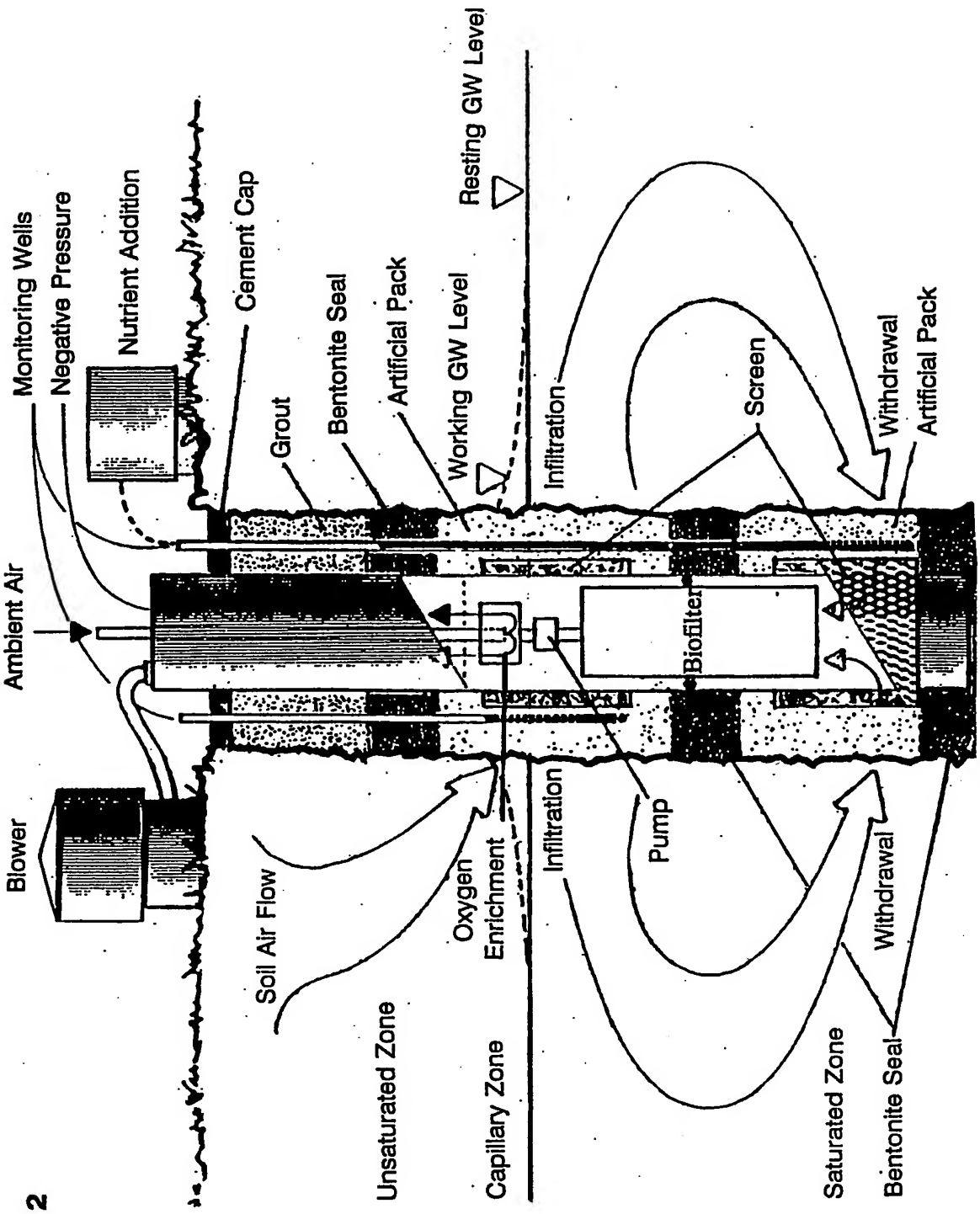

Monitoring Wells
Negative Pressure
Nutrient Addition
Cement Cap
Grout
Bentonite Seal
Artificial Pack
Working GW Level
Resting GW Level
Infiltration
Screen
Withdrawal
Artificial Pack
Ambient Air
Biofilter
Blower
Soil Air Flow
Oxygen Enrichment
Infiltration
Pump
Withdrawal
Unsaturated Zone
Capillary Zone
Saturated Zone
Bentonite Seal

Figure 12

INTERNATIONAL SEARCH REPORT

Inte nal Application No
PCT/US 94/10853

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C02F3/12 C02F3/10 C02F3/34

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C02F

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 475 541 (PREUSSAG NOELL WASSERTECHNIK GMBH) 18 March 1992 see column 5; claims 1-7 see column 1, line 1 - column 3, line 12 see column 4, line 1 - line 16 ---	1,2,5, 8-11,14, 15,17,19
X	EP,A,0 478 097 (VORLOP, KLAUS-DIETER) 1 April 1992 see column 9; claims 1,5-7,13 see column 3; line 39 - line 47 see column 4, line 22 - line 52 see column 5, line 11 - line 19 --- -/--	1,5,8,9, 11,12, 14,15

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 January 1995	10. 01. 95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Teply, J

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INTERNATIONAL SEARCH REPORT

Inter. onal Application No
PCT/US 94/10853

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP,A,0 402 158 (ENVIRONMENTAL SCIENCE AND ENGINEERING, INC.) 12 December 1990 see column 13; claims 1-3,8,13,14 see column 8, line 47 - column 9, line 27 see column 9, line 44 - column 10, line 23 see column 11, line 1 - line 18; figures	1,2,6, 10,14-20
A	DATABASE WPI Week 9132, Derwent Publications Ltd., London, GB; AN 91-235497 & JP,A,3 154 696 (NISHIHARA KANKYO EI) 2 July 1991 see abstract	2,6,7
A	PATENT ABSTRACTS OF JAPAN vol. 15, no. 38 (C-800) (4566) 30 January 1991 & JP,A,02 273 599 (SUSUMU OKADA) 8 November 1990 see abstract	2,6,7
A	EP,A,0 404 466 (UNITED STATES ENVIRONMENTAL PROTECTION AGENCY) 27 December 1990 cited in the application see page 10; claims 1-3 see page 5, line 18 - line 19	1-4,6,10

INTERNATIONAL SEARCH REPORT

Inte. .onal Application No
PCT/US 94/10853

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0475541	18-03-92	DE-A-	4027223	27-02-92
EP-A-0478097	01-04-92	DE-C-	4027218	19-09-91
EP-A-0402158	12-12-90	US-A-	5080782	14-01-92
		CA-A-	2018621	08-12-90
EP-A-0404466	27-12-90	US-A-	5132224	21-07-92
		AT-T-	111953	15-10-94
		CA-A-	2015755	21-12-90
		DE-D-	69012670	27-10-94
		US-A-	5242825	07-09-93